HUMAN MONOCLONAL ANTIBODY TECHNOLOGY.

A tool to investigate human antibody repertoires.

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Veniet tempus, quo ista, quae nunc latent, in lucem dies extrahat et longioris aevi diligentia. Ad inquisitionem tantorum aetas una non sufficit . . . Itaque per successiones ista longas explicabuntur. Veniet tempus, quo posteri nostri tam aperta nos nescisse mirentur.
Seneca Philosophus
Naturales quaestiones, 7, 25, 4-5

Original articles.

The present thesis is based on the following papers which will be referred to in the text by their roman numerals.

I. Ohlin, M., Danielsson, L., Carlsson, R. & Borrebaeck, C. A. K. (1989) The effect of leucyl-leucine methyl ester on proliferation and Ig secretion of EBV-transformed human B lymphocytes. *Immunology* **66**, 485-490.

II. Ohlin, M., Kristensson, K., Carlsson, R. & Borrebaeck, C. A. K. (1992) Epstein-Barr virus-induced transformation of human B lymphocytes: the effect of L-leucyl-L-leucine methyl ester on inhibitory T cell populations. (manuscript in preparation).

III. Danielsson, L., Furebring, C., Ohlin, M., Hultman, L., Abrahamson, M., Carlsson, R. & Borrebaeck, C. A. K. (1991) Human monoclonal antibodies with different fine specificity for digoxin derivatives: cloning of heavy and light chain variable region sequences. *Immunology* **74**, 50-54.

IV. Ohlin, M., Broliden, P.-A., Danielsson, L., Wahren, B., Rosen, J., Jondal, M. & Borrebaeck, C. A. K. (1989) Human monoclonal antibodies against a recombinant HIV envelope antigen produced by primary *in vitro* immunization. Characterization and epitope mapping. *Immunology* **68**, 325-331.

V. Ohlin, M., Hinkula, J., Broliden, P.-A., Grunow, R., Borrebaeck, C. A. K. & Wahren, B. (1992) Human monoclonal antibodies produced from normal, HIV-1 seronegative donors and specific for glycoprotein gp120 of the HIV-1 envelope. (submitted for publication).

VI. Ohlin, M., Sundqvist, V.-A., Gilljam, G., Rudén, U., Gombert, F. O., Wahren, B. & Borrebaeck, C. A. K. (1991) Characterization of human monoclonal antibodies directed against the pp65-kD matrix antigen of human cytomegalovirus. *Clin. Exp. Immunol.* **84**, 508-514.

VII. Ohlin, M., Sundqvist, V.-A., Mach, M., Wahren, B. & Borrebaeck, C. A. K. (1992) Fine specificity of the human immune response to the major neutralizing epitopes expressed on cytomegalovirus gp58/116 (gB) studied by human monoclonal antibodies. (manuscript in preparation).

Contents.

1. Abbreviations.

aa	amino acid(s).
ADCC	antibody-dependent cellular cytotoxicity.
CD	cluster of differentiation.
cDNA	complementary DNA.
CDR	complementarity determining region
CMV	cytomegalovirus.
EBV	Epstein-Barr virus.
gB	glycoprotein B of CMV; contains a complex of gp58 and gp116.
gp41, gp120	membrane glycoproteins of HIV-1.
gp58, gp116	membrane glycoproteins of CMV.
HIV-1	human immunodeficiency virus type 1.
IFN	interferon.
IL	interleukin.
LCL	lymphoblastoid cell line(s).
LeuLeuOMe	L-leucyl-L-leucine methyl ester.
NK cells	natural killer cells.
PBMC	peripheral blood mononuclear cells.
pB1	recombinant protein from HIV-1 gp120 (Putney *et al*., 1986).
PCR	polymerase chain reaction.
penv9	recombinant protein from HIV-1 gp41/120 (Putney *et al*., 1986).
pHM90-5	recombinant protein from CMV gp116 (Meyer *et al*., 1990).
pMbg58	recombinant protein from CMV gp58 (Meyer *et al*., 1990).
pp65	the phosphorylated lower matrix protein of CMV.
SCID-hu mice	SCID mice repopulated with human leucocytes or lymphocytes.
SCID mice	mice with severe combined immunodeficiency.
SDS-PAGE	polyacrylamide gel electrophoresis (according to Laemmli, 1970).
SRBC	sheep red blood cells.
V3	third hypervariable domain of HIV-1 gp120 containing the principal neutralizing determinant.

2. Introduction.

The discovery of antibody molecules and of their ability to specifically recognize particular structures has been of immense importance both for research and for clinical medicine. Serum and serum-derived molecules (which were later identified as antibodies) have long been known to provide protection against toxins and infectious agents (Behring & Kitasato, 1890). Polyclonal serum antibodies of human and animal origin have also been used to transfer such protection between individuals (Behring, 1892; Ordman *et al.*, 1944; Stokes *et al.*, 1944). In addition, specific antibody preparations have been employed as tools in analytical applications (Yalow & Berson, 1959; Engvall & Perlmann, 1971) as well as for purification purposes (Rouslahti, 1976). With the advent of mouse monoclonal antibody technology (Köhler & Milstein, 1975), means became available for obtaining substantially better defined antibody preparations in virtually unlimited quantities. A higher consistency in reagent quality between batches was also achieved. These characteristics have proved important in the further development of analytical and therapeutic applications.

Specific murine monoclonal antibodies against both human target structures and immunogens of non-human origin have been developed in order to obtain reagents suitable for *in vivo* use in humans. The first monoclonal antibody approved as a pharmaceutical was the anti-CD3 monoclonal antibody OKT3, which in clinical studies has proven its ability to prevent rejection of transplants (Cosimi *et al.*, 1981). Treatment of human tumors with antibodies of murine origin has also shown positive results with respect to disease progression (Dyer *et al.*, 1989). Furthermore, antibody-based therapy of infectious diseases is an area in which monoclonal antibodies may replace the polyclonal immunoglobulin preparations used currently. The coupling of drugs, radioisotopes or enzymes to antibodies, and the use of bispecific antibodies may also provide approaches useful in improving the efficacy of antibody-mediated therapy (Raso & Griffin, 1980; Reading, 1983). Administration of murine antibodies to humans for therapeutic or diagnostic purposes, however, is often complicated by the fact that such foreign molecules are recognized by the human immune system. The development of a strong human anti-mouse antibody (HAMA) response (Chatenoud, 1986), potentially limiting the usefulness of the antibody, has been reported in a majority of the studies to date. In addition, the half-life of mouse antibodies in the human circulation is generally short (Khazaeli *et al.*, 1988), implying that repeated high doses of the antibody are required if therapeutic concentrations of the antibody are to be maintained. Furthermore, murine antibodies may be less effective in evoking Fc-receptor-mediated cellular cytotoxicity than in the case of their human counterparts. As a consequence, the attempt has been made to replace the constant domains of mouse antibodies with the corresponding human sequences. Such chimeric antibodies have in several cases proven to be superior to the parent mouse immunoglobulin with respect to effector functions and serum half-life (Heinrich *et al.*, 1989; Liou *et al.*, 1989; Liu *et al.*, 1987; Mueller *et al.*, 1990). Due to the partly successful use of murine monoclonal antibodies, an interest in obtaining similar human antibody reagents arose. Not only do such antibodies suffer

less from certain of the problems associated with murine immunoglobulins when they are used therapeutically, but they can also be developed against specificities not easily recognized by the murine immune system (McCann *et al*., 1988).

Soon after the introduction of mouse monoclonal antibody technology, work was reported describing cell lines which produced specific human antibodies *in vitro* (Steinitz *et al*., 1977; Zurawski *et al*., 1978). Such antibody reagents can be envisaged as being useful not only as analytical and therapeutic reagents, as outlined above, but also as tools for providing insight into the repertoire of human antibody specificities. In order to establish cell lines or other systems producing monoclonal antibodies of human origin or structure, it was necessary to develop methods for finding, manipulating and eventually immortalizing the genes encoding the specific antibodies. Such techniques will be discussed below, together with results obtained in studying the specificity of various human antibodies established from either naive/primary or secondary immune repertoires.

3. Generation and expansion of human B cell repertoires - present technology and future prospects.

The development of systems producing suitable human monoclonal antibodies was long hampered by methodological difficulties (summarized in table 1) normally not encountered in the development of rodent monoclonal antibodies. One of the major problems has been to obtain lymphocytes useful for the generation of human monoclonal antibody-producing cell lines. This has evoked an interest in establishing techniques that can promote the expansion of a preexisting antigen-specific immunoglobulin repertoire or perhaps even generate novel repertoires, as discussed below.

Only in special cases are suitable immune lymphocytes available from human donors following controlled immunization procedures. Furthermore, the lymphocyte source most readily available, i.e. peripheral blood, may not be the optimal source of immune lymphocytes, which after immunization migrate to various lymphoid organs. The majority of circulating B cells are normally high-density lymphocytes which following stimulation produce IgM antibodies (Kuritani & Cooper, 1982), indicating these cells to not have been part of any active immune response to a T-dependent antigen. The low frequency of B cells in peripheral blood that has undergone antigenic selection, isotype switch and hypermutation reduces the possibility of finding and immortalizing the hyperimmune B lymphocytes. In controlled immunization protocol studies using a recall antigen (tetanus toxoid), a rather narrow time period of a few days up to a few weeks following *in vivo* immunization has been shown to be optimal for lymphocyte collection when using peripheral blood as the lymphocyte source (Butler *et al*., 1983; Kozbor & Roder, 1981; Melamed *et al*., 1987), although a low frequency of specific antibody-producing cells can be encountered at other times as well. In some cases, other lymphocyte-containing organs than peripheral blood may be available. However,

since such sources are only rarely available, it may be difficult to find donors with high frequencies of lymphocytes of the specificity desired. Thus, in order to increase the probability of establishing cell lines producing specific human antibodies, steps often have to be taken to specifically enrich the population of interest and sometimes also to manipulate the immune response *in vitro*. A number of techniques have been suggested and designed for this, varoius of them summarized in table 2.

A reasonable approach to increasing the success of immortalization is to raise the frequency of antigen-specific cells in the lymphocyte pool which is to be immortalized, assuming that the process does not reduce the potential of the technique which is employed to immortalize the immune cell. Although rarely used, the depletion of those B cells not internalizing their surface immunoglobulin after the addition of the antigen of interest has been suggested as an approach to enriching antigen-specific B lymphocytes (Kozbor & Roder, 1981). More frequently, however, antibody-producing cells, carrying specific surface immunoglobulins, obtained either directly from PBMC or from EBV-transformed cell lines have been positively selected using antigen immobilized on SRBC (Boylston *et al.*, 1980; Kozbor *et al.*, 1979; Steinitz & Tamir, 1982; Steinitz *et al.*, 1977, 1980, 1988). As an alternative, purification of antigen-specific cells by cell sorting has been attempted, although with limited success (Casali *et al.*, 1986; Kozbor & Roder, 1981). Recently, magnetic bead based technology has been utilized to purify a variety of cell populations. Antigen-specific mouse hybridomas have been selected using this approach shortly after somatic cell fusion, reducing efforts involved in the cloning procedure (Horton *et al.*, 1989; Ossendorp *et al.*, 1989). The selection of mouse x human heterohybridomas by this technique has been unsuccessful, however, due to the absence of surface immunoglobulins on these cell lines (Glaser *et al.*, 1992). The purification of antigen-specific B lymphocytes directly from PBMC (or from LCL) is feasible using this technique, however, and results in a substantial improvement in the yield of specific hybridomas (Glaser *et al.*, 1992). An approach employing the targeted immortalization of antigen-specific mouse lymphocytes has also been used to achieve the same result (Lo *et al.*, 1984; Werkmeister *et al.*, 1991). This technique has not yet been extended to the human monoclonal antibody field, however, and its general usefulness thus remains to be determined.

A major drawback of the above-mentioned selection techniques is their inherent requirement of the surface expression of immunoglobulin. Following activation and differentiation, B cells often lose the expression of these molecules in the cell membrane, while producing and secreting large quantities of antibody. To be able to locate those cells producing the specific antibody, other approaches have to be taken for measuring the activity of the secreted antibody. The possibility of selecting normal B cells on the basis of antibody secretion has been limited previously, due to the poor clonability and survival of normal human B cells in culture even after stimulation with polyclonal activators. Furthermore, only restricted subpopulations of B cells normally respond to these stimuli, thus limiting the expanding repertoire (Kuritani & Cooper, 1982). Recently, three different systems for the growth of B cells *in vitro* have been developed, all of them promoting high frequencies of

activation and in some cases substantial expansion of each clonotype, as reviewed by Gordon (1991). i.) The use of anti-CD3 stimulated and irradiated T-cells + IL-2 has been claimed to activate B cells in larger frequencies than do regular polyclonal activators (Amoroso & Lipsky, 1990; Hirohata *et al.*, 1988). ii.) The cloning of human B-cells in the presence of soluble factors and of feedercells derived from the mouse thymoma cell line EL-4 has been performed successfully. Human B lymphocytes proliferate here to form small colonies that can be screened for immunoglobulin production (Zubler *et al.*, 1985). iii.) Probably the most useful approach to achieving long-term B cell proliferation, however, is one based on recently developed knowledge of factors which induce or prevent apoptosis (programmed cell death) in stimulated B cells. A system involving stimulation through immobilized anti-CD40 monoclonal antibodies and IL-4 has been devised to permit B cell survival and proliferation *in vitro* (Banchereau & Rousset, 1991; Banchereau *et al*, 1991). The prolonged B cell proliferation and immunoglobulin secretion and the high cloning frequency of this system may permit the screening and selection of suitable clones prior to final immortalization of these clones, by use either of regular cell culture techniques or of recently described gene cloning methods (see below).

Due to ethical considerations which restrict possibilities of immunizing humans against most antigens, it has been difficult in many cases to obtain suitable human immune B lymphocytes. Even if immunizations can be performed, the clones that develop may not be of the most beneficial specificity. An approach to circumventing either of these problems would be of great value. Techniques using *in vitro* stimulation/immunization for expanding, and insofar as possible generating, a human antigen-specific immune repertoire outside the human body have been developed in several laboratories (Boerner *et al.*, 1991; Borrebaeck, 1988; Borrebaeck *et al.*, 1988; Carroll *et al.*, 1991; Danielsson *et al.*, 1987; Ho *et al.*, 1985; Hoffmann *et al.*, 1990; Lagacé & Brodeur, 1985; Strike *et al.*, 1984). These techniques are in most cases based on the removal of certain inhibitory cell populations and/or the use of polyclonal stimulation in addition to antigen-specific activation to provide sufficient activation/expansion of the specific B cell population. Some of these approaches have been used successfully to generate cell lines producing specific human monoclonal antibodies (paper III-V; Boerner *et al.*, 1991; Borrebaeck *et al.*, 1988; Ho *et al.*, 1985). The approach developed by Borrebaeck *et al.* (1988) to achieve *in vitro* immunization following removal of inhibitory cell populations by pretreatment of PBMC with lysosomotropic agents, such as L-leucine methyl ester or LeuLeuOMe, has been employed in the present study (paper III-V). This approach not only improves the recovery of antigen-specific plaque-forming cells (Borrebaeck *et al.*, 1988) but also increases the survival and/or proliferation of B cells in culture (paper I; Kristensson *et al.*, unpublished observations) and facilitates subsequent EBV-mediated immortalization of B cells (paper I, II; Wallén *et al.*, unpublished observations). In at least some cases it seems that the antibodies derived by use of these *in vitro* immunization techniques are able to detect determinants which differ from those epitopes which are most immunogenic *in vivo*, thus providing access to a different set of antibody repertoires (see below). This seems to agree with reports

suggesting that specific antibodies which can be detected either with or without previous deliberate *in vivo* immunization frequently seem to recognize different epitopes within the same antigen (Matthes *et al*., 1988; Matthes & Dighiero, 1988). As expected, most antibodies derived from previously unimmunized donors are of the IgM isotype and they have a low affinity for the antigen. Such characteristics are typical of antibodies derived from a naive or primary immune repertoire. IgG-producing clones have been reported recently to occur in some cases (Boerner *et al*., 1991), but the general applicability of this *in vitro* immunization approach to obtaining high-affinity, isotype-switched antibodies from a naive repertoire has to be evaluated further. In addition, whether these IgG-producing cell lines originated from B lymphocytes that underwent class switching (and possibly affinity maturation) during the *in vitro* culture procedure or during a previous *in vivo* response is not currently known. The phenomenon of class-switching has been suggested earlier to occur in a number of murine and human *in vitro* stimulation systems, although no dependence on the presence of antigen has been found (Amoroso & Lipsky, 1990; Banchereau & Rousset, 1991; George & Cebra, 1991; Wen *et al*., 1987). However, utilization of these techniques may prove fruitful in the development of class-switched, antibody-producing cell lines, in particular if it becomes possible to combine them with affinity maturation techniques and the selection of the clones producing high-affinity antibodies.

Little has been done to achieve conditions *in vitro* similar to those of the *in vivo* environment in which the normal immune response develops. However, the activities which direct the ongoing antigen-specific immune response in the germinal centers within the secondary lymphoid organs have recently been elucidated in some detail (Jacob *et al*., 1991; Liu *et al*., 1989, 1991c; MacLennan, 1991). The interactions of antigen-specific B-cells with antigen and antigen-specific T-cells in the microenvironment which accessory cells, such as follicular dendritic cells, as well as accessory stimulatory signals (sCD23, IL-1α, the natural ligand for CD40 etc.) provide seem to supply survival and progression signals to the B cells in an orderly fashion (as reviewed by Liu *et al*., 1992). Such activities have not yet been reproduced entirely *in vitro*. However, dissection of the signals provided by the extrafollicular and follicular/germinal center environment may in the future provide the basis for a complete strategy for achieving antigen-specific immunization of B cells *in vitro*. Stimulation of B cells through the cell surface marker CD40 is known to provide survival signals to the germinal center centrocytes through the activation of the *bcl2* gene (Liu *et al*., 1991b) in much the same way as a number of other survival stimuli signal to the target cell (Henderson *et al*., 1991; Hockenbery *et al*., 1990; Liu *et al*., 1991a, 1991b; Vaux *et al*., 1988). Since stimulation with anti-CD40 antibodies can differentiate centrocytes generated *in vivo* into a memory B cell phenotype (Liu *et al*., 1991a), this mode of differentiation may also be useful for manipulating the differentiation state of B cells cultured *in vitro*. Such manipulations may in some instances be required for example prior to the repopulation of SCID mice (see below) with memory cells. Alternatively, sCD23 and IL1α can be used to differentiate cells into plasma cells (Liu *et al*., 1991a) which can be useful for immunoglobulin mRNA purification. Furthermore, anti-CD40 antibodies

immobilized on CDw32-transfected mouse fibroblasts seem to provide proliferation signals to pure B cells, in addition to the survival and differentiation signals described above (Banchereau & Rousset, 1991; Banchereau *et al.*, 1991; Rousset *et al.*, 1991). To what extent this knowledge may be used to develop antigen-driven *in vitro* culture techniques permitting affinity maturation and antigenic selection remains to be determined. Such an approach will probably require careful selection of the cell populations participating. The different cell populations need probably to be incorporated into the system in some sequential order, each of them promoting certain of the functions such as B cell activation, proliferation, differentiation, selection and survival. Recent studies of human CD4+ subpopulations may serve to identify the useful T cell populations. In particular, the CD45R0+ subpopulation, which has been shown to represent the memory population, provides signals which B-cells require for immunoglobulin synthesis (Clement *et al.*, 1988; Kristensson *et al.*, 1990). The recently described division of human helper T cells into TH1- and TH2-type populations, as defined by lymphokine production profiles (Del Prete *et al.*, 1991a, 1991b; Romagnani, 1991), may also provide insight into the requirements for successful B cell activation *in vitro*. In particular the TH2-type T cell seems to develop in response to soluble antigen and also to mediate effective help for immunoglobulin production in an antigen-specific manner. In contrast, TH1-type clones are less able to promote B cell immunglobulin synthesis, in particular at high T/B cell ratios (Del Prete *et al.*, 1991b). In addition, the lymphokines produced by the murine TH1-type T cells are qualitatively insufficient to collaborate with membrane-derived signals in promoting immunoglobulin synthesis. This implies that specific TH2-derived lymphokines such as IL-4 and/or IL-5 are of importance to antibody production at least by murine B cells (Hodgkin *et al.*, 1991). T-cell clones with such well-defined characteristics as those described above may be used to develop processes encouraging antigen-driven B-cell activation and differentiation. In addition, further studies of the function of other cell populations, such as follicular dendritic cells (Szakal *et al.*, 1988) and regular antigen-presenting cells (Unanue, 1989), as well as of soluble factors, may eventually provide together with systems described above, the theoretical basis for development of technologies designed to promote not only antigen-driven proliferation and immunoglobulin production, but also affinity maturation of antigen-specific antibodies *in vitro*.

However, even with more detailed understanding of the mutual signalling between interacting T and B lymphocytes, certain measures may need to be taken so as to increase the likelihood of interaction between the specific lymphocytes. The low naturally occurring frequencies of these B and T cell populations may otherwise prevent a successful outcome of an antigen-driven procedure. Purification of antigen-specific B lymphocytes may be achieved by using antigen-coated magnetic beads or erythrocytes, as described above. Furthermore, carefully selected antigen-specific T cell clones can be employed as a source of T cell help, as outlined above. Such clones may be specific for any well-defined T cell epitope, which in turn can be covalently linked to the B cell epitope under investigation. Such an approach may circumvent the carrier-induced inhibition of the B cell response to the primary antigen which otherwise may occur (Etlinger *et al.*, 1990). In fact, evidence of the

usefulness of synthetically coupled T and B cell epitopes for the recovery of antigen-specific B cells from the primary repertoire following *in vitro* culture have been obtained recently using a procedure involving immunization of polyclonal B and T cell populations (Chin *et al.*, manuscript in preparation).

Another approach to providing an *in vivo* environment suitable for promoting an antibody immune response is to employ SCID mice repopulated with human lymphocytes (SCID-hu). Due to a lack of functional murine T cells, these mice accept xenografts of human cells (McCune *et al.*, 1988; Mosier *et al.*, 1988). It has also been shown recently that such repopulated mice respond strongly to immunization with a recall antigen (Carlsson *et al.*, 1992; Duchosal *et al.*, 1992; Mosier *et al.*, 1988). This response has many characteristics in common with a normal immune response, for example T-cell dependance, high dose tolerance and dose-dependent selection of B cells which express high affinity antibodies (Carlsson *et al.*, 1992). It has been proposed that only relatively few B cells actually populate the SCID-hu mouse, as evident from the oligoclonal nature of the immunoglobulin produced, thus reducing the B cell repertoire available in such a system (Abedi *et al.*, 1992; Saxon *et al.*, 1991). However, it was shown recently that the removal of NK cells prior to the repopulation of the animal substantially improves the efficiency of this process, implying that a more polyclonal repertoire may be achieved (Carlsson *et al.*, 1992). This agrees with observations of improved engraftment of human thymus in NK- and macrophage-deprived SCID mice (Barry *et al.*, 1991). Furthermore, it seems that early immunization following repopulation is important in order to obtain an antigen-specific immune response, suggesting that antigen-specific activation promotes the survival of the B cell clones of interest in the SCID-hu mouse model. Human B cells which recognize the immunogen can be recovered following immunization, although usually in small amounts, thus providing the starting material for rescuing the immunoglobulin specificity either by conventional cell culture technology or by repertoire cloning (Carlsson *et al.*, 1992; Duchosal *et al.*, 1992). Recent evidence also seems to indicate that primary immunization can be performed in SCID-hu mice and that isotype switching may occur (Abedi *et al.*, 1992; Duchosal *et al.*, 1992; Ifversen *et al.*, manuscript in preparation; Mazingue *et al.*, 1991; Mårtensson *et al.*, manuscript in preparation). This system, in combination with *in vitro* culture techniques described above, may thus in the future provide a general system suitable to initiating proper immune responses involving class switching and possibly also affinity maturation outside the human body.

All of the systems described above for obtaining human antibody-producing cell lines rely on B cell repertoires established in the human lymphocyte donor. This is likely to impose restrictions on the combinations of immunoglobulin genes available (Winter & Milstein, 1991), as a consequence, for example, of clonal deletion (Nossal, 1991). Recently, however, a system designed for the development of human humoral immune repertoires through the recombination of human germ line sequences in animals, that may circumvent some of these restrictions, was described. Mice that are transgenic for the unrearranged human immunoglobulin heavy chain locus have been shown to carry mouse B cells which express functional human

immunoglobulin (Brüggemann *et al.*, 1989, 1991). Such a system may provide an environment permitting the development of a human antibody repertoire which differs from the repertoire provided in the human body. Alternatively, techniques to differentiate human B cell precursors *in vitro* may be developed in order to create human naive antibody repertoires. Systems developed earlier for delivering the signals necessary to differentiate murine B cell precursors may be taken as a starting point in developing this type of technology for human cells (Kinashi *et al.*, 1988, 1990; Sudo *et al.*, 1989). Such naive repertoires generated *in vitro* may possibly contain heavy and light chain combinations which, due to clonal deletion and preferential expansion of other specific clones, are not frequently available in a lymphocyte population established *in vivo*. If such lymphocytes generated *in vitro* could be used together with appropriate autologous T cells (or T cell clones) to repopulate SCID mice, it might be possible to generate novel secondary immune repertoires through an immunization procedure similar to the one described above which utilizes lymphocytes generated *in vivo* as the source of an immune repertoire.

In contrast to the models described above, which rely entirely on the complete immunoglobulin repertoire which is formed within existing lymphocytes, genetic engineering now provides a powerful tool for developing and screening entirely new immune repertoires. This approach may thus eliminate some of the constraints discussed above which are built into the cellular systems. Such techniques are all based on the cloning of large repertoires of immunoglobulin variable region sequences into expression vectors (Barbas *et al.*, 1991; Chang *et al.*, 1991; Clackson *et al.*, 1991; Huse, 1991a; Huse *et al.*, 1989; Marks *et al.*, 1991; McCafferty *et al.*, 1990). Selection of clones expressing a specific antibody can be made with the help of regular immunological detection systems, following steps (panning or affinity chromatography) designed to enrich for genes expressing antigen-specific immunoglobulin specificities (Barbas *et al.*, 1991; Clackson *et al.*, 1991; Marks *et al.*, 1991). Such systems have been used recently to establish panels of human monoclonal antibody fragments from phage display libraries (Burton *et al.*, 1991; Clackson *et al.*, 1991; Marks *et al.*, 1992; Mullinax *et al.*, 1990; Persson *et al.*, 1991). It has been demonstrated that large repertoires of variable region combinations which recognize a particular antigen can be isolated. The potential for establishing antibody-producing systems containing light- and heavy-chain combinations not normally found *in vivo* has been discussed (Clackson *et al.*, 1991). Recent experiments have shown that this system, involving the screening of large combinatorial libraries following antigen-specific selection, provides a technology able to handle the very large numbers of clonotypes that need to be studied in order to find those rare gene combinations encoding antigen-specific antibodies in libraries established from unimmunized individuals (Marks *et al.*, 1991). Apart from the efficiency of this technology in detecting the clones producing a specific antibody, another advantage of this technique is that it provides easy access to other procedures requiring prior cloning of the immunoglobulin variable domain gene sequences. The possibility of changing and possibly improving antibody-binding characteristics by use of this technology, through exchanging the light chain of the original antibody for other light chains present in the light chain variable region

library, has been put to practical use. It has been shown that a number of new, high-affinity, anti-phenyloxazolone, heavy and light chain combinations could be identified with this approach (Clackson *et al.*, 1991). The use of site-directed, random mutagenesis of immunoglobulin genes which encode a specific antibody and the subsequent expression of these genes in a phage display library has also been shown to provide an approach to developing and expanding a repertoire of antibodies of differing affinities for the original antigen (Barbas, 1992; Huse, 1991b). Alternatively, it has been proposed that through developing transgenic mice which express the rearranged immunoglobulin genes, antigen-driven hypermutation and clonal selection of murine immunoglobulin coding transgenes can be obtained in an *in vivo* environment (Conrad *et al.*, 1991; O'Brian *et al.*, 1987). This technique could obviously be applied to the affinity maturation of human immunoglobulin genes in a similar manner. Altogether, these systems can provide powerful tools for developing a wide variety of human monoclonal antibody specificities, and in the near future they may largely replace the current technology which is dependent upon cellular biology (Winter & Milstein, 1991).

4. Immortalization of immunoglobulin specificities - basic technologies.

Due to the poor potential of human B cells as compared with T cells, to proliferate over extended periods of time in culture, efforts have to be made to rescue the immunoglobulin phenotype by artificial means. In initial attempts, human B cells were immortalized with Epstein-Barr virus, a human herpesvirus which specifically infects human B lymphocytes. Some of the infected cells are transformed and show a virtually unrestricted capacity to proliferate in culture (Henle *et al.*, 1967; Pope *et al.*, 1968), in the process also being induced to synthezise and secrete human antibodies (Rosén *et al.*, 1977). In further developments, the use of somatic cell fusion (which is the standard immortalization procedure used in murine monoclonal antibody technology) for the establishment of human monoclonal antibody-producing cell lines was introduced (Croce *et al.*, 1980a; Olsson & Kaplan, 1980). The combination of these techniques, i.e. EBV-transformation and somatic cell fusion, represented an important improvement in the efficiency with which cell lines producing human monoclonal antibodies could be established (Kozbor *et al.*, 1982). This approach combined the potential to expand specific cells prior to fusion, with improved possibilities for prescreening clones for antigen-specific reactivity prior to fusion, as well as with an increase in fusion frequency (Kozbor & Roder, 1984). Often, however, the human fusion partners available (as reviewed by Kozbor & Roder, 1983) were not optimal due to their low fusion frequencies, the expression of endogenous antibody chains which contaminated the final product, and low levels of antibody secretion. The utilization of mouse myelomas has been successful to some extent, although the hybrids are often unstable due to the loss of human chromosomes. In particular chromosome 2, which harbours the human κ chain locus is frequently lost from such human x mouse hybrid cell lines (Croce *et al.*, 1980b). However, the development of human x mouse fusion partners has provided a suitable tool for establishing more stable trioma cell lines producing human

monoclonal antibodies at a high level (Östberg & Pursch, 1983). The approach, using EBV-transformation followed by somatic cell fusion of LCL to human x mouse heterohybridomas, has been used in the experiments described below for developing cell lines producing human monoclonal antibodies from lymphocytes obtained from previously non-immunized (paper III-V) or immunized (paper VI, VII) individuals (table 3).

4.1. EBV-induced transformation of human B lymphocytes.

As described above, EBV infects human B lymphocytes and transforms them into immortal, immunoglobulin-producing cell lines. The complicated intracellular processes which eventually result in the transformation of a part of the B cell population following primary infection *in vitro* have been reviewed recently (Alfieri *et al.*, 1991; Mellinghoff *et al.*, 1991; Middleton *et al.*, 1991; Thorley-Lawson, 1988). This immortalization approach has been very useful in human monoclonal antibody technology, since it allows relatively efficient immortalization of B cells, and a number of stable antibody-producing cell lines have been established by use of this method (James & Bell, 1987). However, problems related to the commonly occurring instability of these cell lines are encountered frequently. In addition, the technique is not always applicable since only a minor part of the B cell pool is susceptible to this immortalization procedure, thus not only reducing the probability of the rare specific B lymphocytes being found, but also possibly restricting the repertoire that can be obtained with this technique. However, since virtually every available immortalization technique has some restriction on the B cell populations which it can efficiently immortalize, EBV-immortalization may actually complement the other technologies which are available for rescuing the production of antigen-specific antibodies.

If the EBV-immortalization technology is to be useful, it is important to know which target B cell populations are sensitive to the virus, since this may effect the procedures that should be used for manipulating the lymphocyte populations prior to immortalization. It was suggested initially that the population most efficiently immortalized by the virus was primarily that of high-density B cells, seen as representing resting B lymphocytes (Åman *et al.*, 1984), and furthermore that the virus receptor (CD21 or CR2) was lost shortly after activation *in vitro* (Stashenko *et al.*, 1981). It was also shown that entry into the cell cycle potentially reduces the ability of EBV to transform the B lymphocyte (Roome & Reading, 1987) and that some stimulated cells may be unable to convert the linear EBV-genome into a circular form following infection, thus preventing the development of an immortal phenotype (Hurley & Thorley-Lawson, 1988). Such studies indicate that serious problems may be encountered whenever the attempt is made to use EBV-infection as the immortalization approach following an *in vitro* immunization/stimulation technique of whatever type is selected. Later studies, however, have indicated that partially activated cells may be the population most effectively immortalized (Chan *et al.*, 1986; Crain *et al.*, 1989). In the approach we employ, short term activation has

actually been found to slightly enhance the EBV-induced transformation frequency (Wallén *et al.*, unpublished observations). It is probable that the mode of stimulation selected has profound effects on the ability of EBV to subsequently immortalize *in vitro* cultured B cells. It thus seems that human B cells proliferating in response to immobilized anti-CD40 antibody and IL-4 retain their ability to be immortalized by the virus (Banchereau *et al.*, 1991). Furthermore, although some human lymphocytes are not immortalized by EBV-infection, they may frequently be activated and committed to short-term antibody production. Such cells may be identified by sensitive screening procedures and be immortalized by other means. Highly efficient immortalization procedures based on microfusion (Foung & Perkins, 1989; Foung *et al.*, 1990b; Glaser *et al.*, 1989; Ohlin & Borrebaeck, 1992; Perkins *et al.*, 1991), or on PCR amplification of the immunoglobulin variable region genes (Larrick *et al.*, 1989a, 1989b) followed by repertoire cloning provide means of rescuing the specificities of such cells. Approaches of these types may also be taken to rescue those lymphoblastoid cells which tend to differentiate into non-proliferating plasma cells producing antibody at a high rate (Azim & Crawford, 1988; Wendel-Hansen *et al.*, 1987).

With respect to immortalization efficiency, a commonly occurring problem is the existence of EBV-specific or non-specific cytotoxic activities among human lymphocytes. EBV has been found to establish latent infections at very high frequency in human populations. As a consequence, most lymphocyte donors are likely to have EBV-specific T lymphocytes which inhibit the outgrowth of LCL (Moss *et al.*, 1978; Sugamura & Hinuma, 1980; Thorley-Lawson, 1980; Thorley-Lawson *et al.*, 1977). Furthermore, it has been suggested that large granular lymphocytes (NK-cells) (Kuwano *et al.*, 1986; Masucci *et al.*, 1983) as well as α- and γ-IFN (Andersson *et al.*, 1985c; Menezes, 1976; Thorley-Lawson, 1981) reduce the outgrowth of EBV-transformed B cells as well as their immunoglobulin production. Stimulatory signals provided to NK-cells and to T-cells by EBV-infected B cells induce IFN production, reducing the outgrowth potential of LCL (Andersson *et al.*, 1984; Andersson *et al.*, 1985a; Lotz *et al.*, 1986). Furthermore, it has been shown that PBMC, which are particularly resistant to EBV-transformation, produce substantially higher amounts of IL-2 and γ-IFN following EBV-infection than do other PBMC. The ability of anti-IL-2 and anti-γ-IFN to block this potent outgrowth inhibition implies an important role of these cytokines in the downregulation of EBV-transformation *in vitro* (Gosselin *et al.*, 1989).

A number of approaches have been employed for curtailing the activities which inhibit the outgrowth of B cells which have recently been infected with EBV. These include the removal of T cells by rosetting with SRBC (Moss *et al.*, 1978), the inhibition of T-cell activity by use of cyclosporin A (Andersson *et al.*, 1985b; Bird *et al.*, 1981; Pereira *et al.*, 1983; York & Qualtiere, 1990) and B cell purification through use of immunoadsorbent chromatography (Thorley-Lawson *et al.*, 1977) or antibody-coated magnetic beads (Rasmussen *et al.*, 1992). We have reported that pretreatment of PBMC with the lysosomotropic agent LeuLeuOMe, in addition to the toxic effects it has on monocytes, cytotoxic T lymphocytes and NK cells (paper

I; Thiele & Lipsky, 1986), also reduces the potential of the cell population to inhibit outgrowth of EBV-transformed B cells (paper I, II). Similar results for the curtailing of outgrowth inhibition through use of the LeuLeuOMe-precursor L-leucine methyl ester were obtained in an independent study (Schultz *et al.*, 1989). The dipeptide ester, which mediates its cytotoxic activity through a polymerized derivative formed by the action of the enzyme dipeptidyl peptidase I, which is present in the lysosomal compartment (Thiele & Lipsky, 1990), has been shown earlier to remove cytotoxic human CD4+ and CD8+ T cells active in e.g. graft versus host disease (Thiele *et al.*, 1987). However, previous estimates of the frequency of EBV-specific cytotoxic T cells in PBMC (Moss *et al.*, 1978) suggested the inhibitory activity which was observed to not reside in the T cell population. It was thus proposed that the cell population active in inhibiting the outgrowth of LCL was contained within the NK cell population (paper I). However, no evidence for inhibitory activity in the CD16+ NK-cell population was obtained when FACS-purified NK cells were added to EBV-infected lymphocytes treated with LeuLeuOMe or to purified infected B-cells (paper II). These experiments do not completely rule out the possible involvement of NK-cells in the delivery of signals which mediate outgrowth inhibition, but it implies that NK cells are not necessary for the inhibitory activity, which could be reconstituted through the addition of other cell populations (see below). Additional studies using purified NK-cell subpopulations, such as the HLA^{low}, CD11b$^{very\ high}$, CD16$^{very\ low}$ population which has been shown to represent a killer NK phenotype (Lebow & Bonavida, 1990), may show further whether NK cells are at all involved in the inhibition of the clonal outgrowth detected in this system.

As pointed out above, T cells from EBV-infected donors have been known to exert potent inhibitory activities on autologous EBV-transformed B lymphocytes. The frequency of such cells in peripheral blood was, as indicated above, believed to be too low to account for the inhibition (paper I). However, the frequency of EBV-specific cytotoxic lymphocytes found is highly dependent upon the assay methodology employed, and reports of the occurrence of high frequencies of such cells among PBMC have been published (Bourgault *et al.*, 1991; Rickinson *et al.*, 1981), in agreement with results of our own reconstitution experiments. These studies show that both CD4+ T cells and CD8+ T cells (but not LeuLeuOMe-treated CD4+ T cells) are able to inhibit the outgrowth of LCL under the conditions employed in these experiments (paper II). Also, it has been shown that several CD8+ subpopulations including both CD11b+ (associated with suppressor activity) (Landay *et al.*, 1983) and CD11b- cells, are affected by LeuLeuOMe treatment (paper II). Similarly, cytotoxic effects by LeuLeuOMe on diverse CD4+ subpopulations, such as CD45RA+ and CD45R0+ cells (representing naive and memory T cells, respectively), have been shown to remove parts of these populations, without preferentially affecting any of them (paper II). This appears to agree with previous observations indicating mouse CD4+ T cells to be functionally impaired by LeuLeuOMe (Mowat & Leck, 1990), and partly contradicts the assertion earlier that CD4+ T cell populations (except the cytotoxic CD4+ T cells) are unaffected by the dipeptide ester (Thiele & Lipsky, 1986). Furthermore, it was shown that the induction of γ-IFN mRNA in CD4+ cells in response to EBV-infected B cells was

substantially delayed in a LeuLeuOMe-treated population (paper II). The potential of a CD4+ T cell population for promoting outgrowth inhibition through the secretion of γ-IFN (Thorley-Lawson, 1980) may thus be substantially reduced following LeuLeuOMe treatment. Whether this treatment functions through partially or completely removing γ-IFN-producing cells, through activating the production of other components (such as IL-10-like activity (Moore *et al.*, 1990)) which are known to inhibit γ-IFN-production, or through downregulating T cell activation generally, has not been determined, although T cell activation, as measured by IL-2 mRNA production, did not seem to be affected. Apart from producing outgrowth inhibitory cytokines, CD4+ cells may mediate direct cellular cytotoxicity against autologous LCL (Chen *et al.*, 1988; Misko *et al.*, 1991). Further studies will thus be needed to determine conclusively whether the outgrowth inhibitory effect exerted by the CD4+ T cell population is mainly mediated by soluble factors or by direct cell-to-cell contact.

4.2 Other techniques to immortalize the genetic information encoding human immunoglobulins.

Apart from the classical immortalization technologies described above, i.e. somatic cell fusion and EBV transformation, a few other techniques have been developed to immortalize human B lymphocytes. Some reports have described the transfection of cellular DNA obtained from immortal cell lines into murine and human B cells as a potentially useful immortalization approach (Abken *et al.*, 1988; Jonak *et al.*, 1984, 1988). As of yet these techniques have had very little practical importance in human monoclonal antibody technology, however. Future developments will need to show whether these technologies can be useful for the immortalization of human B cells.

Recently, several laboratories have utilized gene cloning and expression technologies to immortalize human antibodies. Such approaches may be utilized not only to establish antibody repertoires, as described above, but also to rescue unstable cell lines producing human monoclonal antibodies. The methodology of amplifying the variable sequences of the immunoglobulin gene by PCR using degenerate primers (Larrick *et al.*, 1989a, 1989b; Orlandi, 1989) provides a powerful immortalization technology. The possibility of combining this approach with combinatorial library techniques and/or with site-directed mutagenesis further underlines the importance of this approach (see above).

5. Repertoires of antibody specificities.

During the maturation of human B cell precursors, immunoglobulin heavy and light chain variable germ line genes rearrange to form the mature genes (Brack *et al.*, 1978; Maki *et al.*, 1980a, 1980b). Variability in the rearranged genes is achieved not only by selection of different combinations of V, D (heavy chain only) and J genes, but also through junctional deletions and N-region insertions. B cell precursors with

successfully rearranged heavy and light chain genes, which allow the production of functional immunoglobulin molecules eventually emerge as mature B lymphocytes. The deletion of certain self-reacting clones, however, imposes certain restrictions on the B cell repertoires available (Nossal, 1991). The mature B cells reach the circulation and the peripheral organs, but unless they are properly stimulated with antigen and recieve appropriate T cell help they rapidly die (De Freitas & Coutinho, 1981). These naive B lymphocytes, together with previously activated cells, memory cells and long-lived self-renewing CD5+ B cells constitute the repertoire available at any given time.

The molecular characteristics detectable in selected antigen-specific clones in naive as well as primary and later repertoires have been studied in great detail in the mouse system using well-defined antigens such as phenyloxazolone and 4-hydroxy-3-nitrophenylacetyl. In principle, three types of antigen-recognizing antibodies are produced following immunization, namely (i) low affinity antibodies (mainly IgM) derived from unmutated germ line genes; (ii) medium affinity antibodies showing no evidence of affinity maturation caused by somatic mutation; and (iii) medium and high affinity antibodies (mainly IgG) which show evidence of somatic mutation of the original germ line genes (Berek & Milstein, 1988). The first type, the low-affinity antibodies, can be isolated at all times, even from naive unimmunized animals, and they typically show extensive cross-reactivity to unrelated antigens, as outlined further below. The other two antibody types expand following immunization with a T-dependent immunogen, the latter type appearing during the later stages of the primary immune response. Following immunization, germinal centers populated with oligoclonal antigen-specific B cells develop (Kroese *et al.*, 1987). In this environment, clonal expansion of the specific cells accompanied by extensive somatic mutation occurs. Sequential mutations within each clone (Jacob *et al.*, 1991; Kocks & Rajewski, 1988) greatly enhance the variability available. Cells maintaining antigen-binding properties are rescued from apoptosis, which would otherwise occur in the light zone of the germinal center (Liu *et al.*, 1991a, 1991b). These hypermutated, antigen-binding cells may then receive signals to differentiate into memory or plasma cell phenotypes, as described in section 3. Upon restimulation with the same antigen the memory population together with other newly formed clones may be recruited for antibody production and possibly undergo further hypermutation (Berek & Milstein, 1988). Following such restimulation, expansion of originally rare clones that may have some competetive advantage such as higher affinity for the antigen or advantageous reaction kinetics may take place and result in shifts in the clonotypes dominating the antibody repertoire (Berek *et al.*, 1985; Foote & Milstein, 1991).

Antibody monospecificity is a property which is not easily determined, the outcome of such an investigation being highly dependent on the analytical procedure used to evaluate this particular property. However, antibodies recognizing a multitude of seemingly unrelated antigens have been found in the human serum immunoglobulin fraction (Guilbert *et al.*, 1982). All three major isotypes of serum immunoglobulins have been shown to contain multispecific antibodies. These antibodies not only

recognize foreign antigens, but frequently cross-react with human autoantigens such as DNA, structural proteins, IgG, thyroglobulin and insulin, which indicates that they represent a group of autoantibodies. The basis for the unexpected multireactivity pattern is not entirely clear, although the observed crossreactivity of one antibody which recognizes both a bacterial polysaccharide and polynucleotides has been suggested to be due to charged groups which are similarly spaced in these antigens (Kabat *et al.*, 1986). In general, multireactive B cells exist in similar frequencies in normal and autoimmune individuals, and the antibodies produced are generally low affinity antibodies of the IgM isotype. In addition, high frequencies of B cell clones which produce high-affinity monospecific autoantibodies are found among lymphocytes from autoimmune patients but not among lymphocytes obtained from healthy individuals. Such high affinity antibodies are possibly involved in the autoimmune condition (Burastero *et al.*, 1988; Nakamura *et al.*, 1988b). The appearance of clones producing multireactive antibodies represents a problem in monoclonal antibody technology, and a number of established murine hybridomas have been shown to secrete such polyreactive antibodies (Dighiero *et al.*, 1983; Fox & Siraganian, 1986). Similarily, the screening of monoclonal cell lines which originate from lymphocytes obtained from normal individuals and from patients suffering from immunoproliferative or autoimmune diseases has indicated that approximately 5-20% of the clones produced polyreactive antibodies (Avrameas *et al.*, 1983; Nakamura *et al.*, 1988b; Vanderslice *et al.*, 1988). Whenever rare and specific clones have to be identified, such multireactive clones represent a major problem, one which that has to be solved early in the screening procedure if a high efficiency of the procedure is to be ensured.

Among B lymphocytes isolated from fetal tissue, a major portion of the population expresses the T cell marker CD5. This antigen is also present on a minor population of adult B cells. In limiting-dilution experiments multi- and autoreactive antibodies have been found to be produced mainly though not exclusively by cells of this CD5+ B cell population, as reviewed by Casali & Notkins (1989). In fact a majority of CD5+ B cells seem to be committed to the production of polyreactive antibodies (Casali *et al.*, 1987; Nakamura *et al.*, 1988a). Studies of CD5+ B lymphocyte immunoglobulin genes indicate an overrepresentation of certain VH region genes, in particular from the VHIV, VHV and VHVI gene families, which are located close to the constant-region genes in the unrearranged heavy chain locus (Sanz *et al.*, 1989a; Schutte *et al.*, 1991). This overrepresentation is similar to the gene expression observed in CD5+ neoplastic B-CLL (chronic lymphocytic leukemia), as well as in the early B-cell repertoire (Alt *et al.*, 1987; Humphries *et al.*, 1988). Several reports also indicate that cells producing polyreactive antibodies express variable region genes that are very similar, or identical, to sequences identified in the germline (Dersimonian *et al.*, 1989; Harindranath *et al.*, 1991; Sanz *et al.*, 1989a, b). Specific characteristics of the D-segments have also been suggested to be a factor involved in determining antibody polyreactivity (Harindranath *et al.*, 1991). Similarily, a difference in length and sequence has been reported between heavy chain CDR3 in polyreactive, natural antibodies and induced, monospecific antibodies established from the mouse antibody repertoire (Chen *et al.*, 1991; Wang *et al.*, 1991).

However, recent studies have neither conclusively confirmed a difference in VH gene family usage between the total immunoglobulin pool, a pool of non-crossreactive antibodies and a pool of multispecific antibodies (Guigou *et al.*, 1990, 1991), nor provided any support for the suggestion that the CD5+ B cell population is the main source of clones producing autoantibodies, many of which may be polyreactive (Vernino *et al.*, 1992). Further studies will be needed to resolve issues of defining the cell populations containing the repertoire of polyreactive antibodies, so as to ensure that suitable steps can be taken to minimize the problems associated with the clones producing multireactive immunoglobulins.

The frequently occurring polyreactive nature of IgM antibodies obtained from the natural/naive immune repertoire suggests that IgM antibodies raised from individuals which have not been immunized *in vivo* prior to the collection of lymphocytes may potentially be crossreactive. As regards some of the data presented above, it has been suggested that the removal of the CD5+ B cell population prior to stimulation or immortalization may eliminate a major portion of the multireactive clones. Although this approach has been used only rarely several monospecific IgM antibodies have been obtained through employing proper screening techniques and eliminating multireactive clones following crossreactivity testing (papers III-V; Chin *et al.*, manuscript in preparation). Four such antibodies have been sequenced and they fail to show any strong overrepresentation of VH-gene families connected with multireactive immunoglobulins (paper III, Larrick *et al.*, 1989a). Among digoxin-reacting antibodies, one of the three clones produces a heavy chain which is encoded by a virtually unchanged germ-line V-region sequence, but it is not known whether this clone originates from a CD5+ or from a CD5- B lymphocyte. The other two digoxin-specific antibodies were found to deviate to a larger extent, however, from the human germ line sequences reported earlier. Whether they originated from B lymphocytes previously stimulated by other, unknown, cross-reacting antigens, or represent previously uncharacterized germ line sequences is not known. Although these digoxin-specific antibodies all recognize the antigen with low affinity (paper III), they do not show any detectable multireactivity, as shown in figure 1. Similarly, monospecific antibody fragments can be obtained from combinatorial libraries expressed in a phage display system and derived from nonimmunized (seronegative) donors (Marks *et al.*, 1991), suggesting the usefulness of the naive repertoire in the generation of cell lines producing specific antibodies. The low affinity of such antibodies, however, frequently requires that additional steps to enhance their antigen-binding properties be taken so as to ensure a suitable reagent quality. The development of *in vitro* (Barbas, 1992; Huse, 1991b) or *in vivo* (Conrad *et al.*, 1991; O'Brian *et al.*, 1987) affinity maturation techniques may in the future provide tools which can be used to accomplish such improvements in the original, low affinity antibodies.

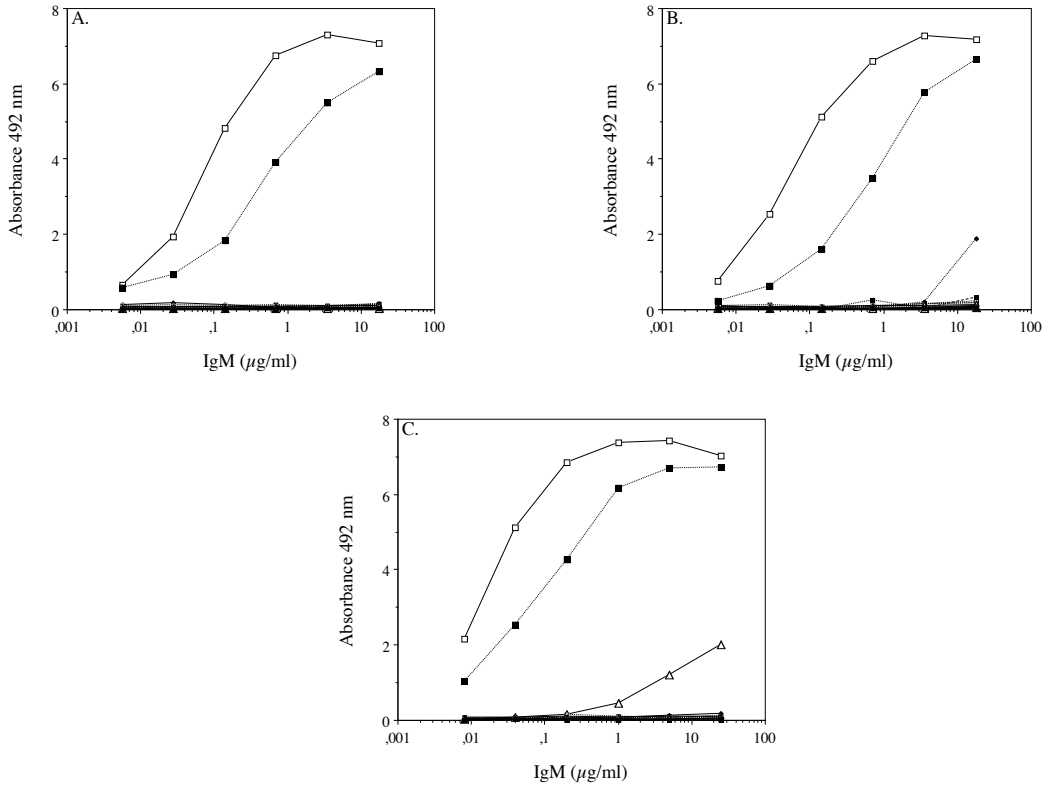

Figure 1. Reactivity of human anti-digoxin monoclonal antibody MO6 (A), LH92 (B) and LH114 (C). Substantial reactivity was only found against digoxin-bovine serum albumin (❒) and digoxin-transferrin (■) conjugates in this assay. A minor reactivity against a DNP-human serum albumin (Δ) conjugate was detected for LH114 (at approximately 1000 times higher antibody concentration). No reactivity was found against bovine and human serum albumin, thyroglobulin, collagen, insulin, keyhole limpet hemocyanin, ovalbumin, RNA or single- and double-stranded DNA.

6. Humoral immune response against some HIV-1 related proteins.

Human immunodeficiency virus type 1 (HIV-1) is one of the RNA viruses of the lentivirus subfamily of retroviruses, which are the causative agents of acquired immunodeficiency syndrom (AIDS) and related clinical disease complexes (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). The virus generally infects the human helper T cell population following its adherence to the cell surface molecule CD4. Other cell targets, such as monocytes/macrophages and brain microglial cells (Price *et al.*, 1988) may also be infected. Immediately following infection, a viremic state occurs, but after sero-conversion the level of free virus drops dramatically and a latent infection stage develops. Eventually, the immune defense of the host is impaired as a consequence of the infection, resulting for example in a substantial reduction in the CD4+ T cell level and in immune system malfunction (Fauci *et al.*, 1984; Gottlieb *et al.*, 1983), a situation paving the way for tumor development and deleterious opportunistic infections. Furthermore, neurological manifestations caused by the virus may effect the health status of the infected individuals.

Because of the current inability to cure HIV-infected individuals, and the severity of the sequelae which occur as a consequence of the infection, substantial efforts to develop new treatment approaches are in progress. The use of anti-viral drugs such as AZT (3'-azido-2',3'-dideoxythymidine) (Mitsuya *et al.*, 1985) which affect the activity of virus-specific enzymes, have proven their ability to induce clinical improvement in the patients treated (Yarchoan *et al.*, 1986, 1987). The potential role of immunological surveillance to prevent infection has also evoked an interest in the development of vaccines which can stimulate cellular and humoral protective immunity (Redfield *et al.*, 1991). Despite initial failure to confer protection against the virus through use of antibody preparations (Prince *et al.*, 1988), recent experiments show mono- and polyclonal antibody preparations to actually provide either protection against infection or delay of infection by HIV-1 (Emini *et al.*, 1990, 1992; Prince *et al.*, 1991). This implies the importance of evaluating different types of HIV-specific antibodies which may be included in or induced by reagents used for passive immunotherapy and vaccination, respectively. Studies of antibodies (polyclonal and monoclonal preparations) can provide such information as well as information on the humoral immune responses induced by the virus *in vivo*.

Antibodies mediating several types of activities directed against the virus *in vitro*, such as neutralization, ADCC, the inhibition of syncytia formation and the inhibition of viral spread between cells, have been identified. The neutralizing activities of polyclonal sera and virus-specific antibodies obtained from infected and immunized individuals and animals have been investigated extensively, as reviewed recently (Goudsmit *et al.*, 1991; Nara *et al.*, 1991). Several epitopes on gp41/120, in particular within the V3 region of membrane glycoprotein gp120, have been shown to elicit type-specific, neutralizing antibodies *in vivo*. A number of other minor neutralization epitopes have been identified through use of immunoaffinity purification of such antibodies on immobilized peptides, or through induction of virus-neutralizing antibodies following immunization with synthetic peptides covering such epitopes (Cease, 1990). A conformational, isolate-crossreactive neutralization epitope has also been located in the vicinity of the CD4-binding site of gp120, and a number of neutralizing human monoclonal antibodies have been shown to interact with this epitope (Ho *et al.*, 1991; Posner *et al.*, 1991; Thali *et al.*, 1991). Furthermore, carbohydrate structures on the virus envelope are known to be recognized by virus-neutralizing antibodies (Hansen *et al.*, 1990, 1991). Other functional activities, such as ADCC, are known to be mediated by polyclonal sera and by certain monoclonal antibodies (including some neutralizing antibodies), such as those which recognize the major neutralizing epitope within the V3 region of gp120 (Broliden *et al.*, 1990). The study of ADCC activity referred to here has utilized a monoclonal antibodies of murine origin. The exchange of mouse- for human-constant domains of a monoclonal antibody has been shown to induce the capacity of mediating ADCC activity through human effector cells (Liou *et al.*, 1989). Differences between human and murine antibodies in their ability to mediate such functional activities, as well as possible differences in the antibody repertoire evoked in experimentally immunized animals and infected individuals, suggest that

further studies of the human humoral immune response against HIV-1 related epitopes will contribute information useful in the design of therapeutic modalities.

6.1. Antibodies against HIV-1 gp120, in particular those recognizing the V3 region.

A wide variety of epitopes recognized by antibodies raised by native or recombinant gp120, or by peptides derived from that molecule, have been identified (see Cease, 1990 for a review). The V3 region of HIV-1 gp120 has been shown to be a major target for neutralizing antibodies (Goudsmit *et al.*, 1988; Javaherian *et al.*, 1989; Kenealy *et al.*, 1989; Palker *et al.*, 1988; Rusche *et al.*, 1988). This principal neutralizing determinant is part of one of the disulphide-linked loop structures of the protein (Leonard *et al.*, 1990). This loop, although highly variable between different strains and clinical isolates, frequently (in 237 of 245 studied cases) carries a conserved sequence (gly-pro-gly) at its tip. Furthermore, some of the amino acids adjacent to these three residues are also highly (≥80%) conserved, and as a whole this sequence matches a type II β-turn (LaRosa *et al.*, 1990). Despite the conserved nature of the tip of the V3 loop, polyclonal antisera and a variety of murine and human monoclonal antibodies recognize this epitope in a type-specific manner (Goudsmit *et al.*, 1988; Javaherian *et al.*, 1989; Matsushita *et al.*, 1988; Palker *et al.*, 1988; Profy *et al.*, 1990; Scott *et al.*, 1990).

During the evolution of the humoral immune response in an infected individual, the neutralizing activity which develop initially recognizes the V3-loop in a type-specific manner (Nara *et al.*, 1991). These neutralizing antibodies select for neutralization escape mutants carrying mutations which cause amino acid substitution either within the V3 region or in other parts of the molecule which affect the exposure of the V3-domain (Masuda *et al.*, 1990; Nara *et al.*, 1990; Wahlberg *et al.*, 1991). Such mutants eventually induce other B cell clones to produce other V3-specific neutralizing antibodies. In this manner, the entire V3-specific antibody repertoire may recognize and neutralize a more diverse set of virus isolates. Similarly, sequential immunization with recombinant non-glycosylated gp120 preferentially induces B cell clones which produce cross-reacting antibodies in mice (Klinman *et al.*, 1991). Whether the antibodies produced by these clones actually detect the conserved parts of the V3-loop, or detect some other conserved epitopes, is not currently known. It has been established that some cross-neutralizing antibodies may detect epitopes different from those of the V3 region, such as those of the CD4-binding domain (Ho *et al.*, 1991; Kang *et al.*, 1991; Thali *et al.*, 1991), although the exposure of this epitope may require the use of native, glycosylated antigen during the immunization procedure (Haigwood *et al.*, 1992; Ho *et al.*, 1991). Furthermore, the establishment of polyclonal antisera and the generation of cell lines producing V3-specific antibodies which recognize a variety of HIV-1 strains (Boudet *et al.*, 1992; Javaherian *et al.*, 1990; Laman *et al.*, 1992; Ohno *et al.*, 1991; Åkerblom *et al.*, 1990), imply that a cross-neutralizing response can be obtained towards the conserved parts of the variable V3-region. In view of these findings and also the

partially protective effect of V3-specific antibodies against virus challenge in chimpanzees (Emini *et al.*, 1990, 1992), as well as the correlation shown between certain V3-specific antibodies and protection against virus transfer to children born to HIV-infected mothers (Devash *et al.*, 1990; Rossi *et al.*, 1989), passive immunization of human subjects with V3-specific antibodies may be of clinical value. These antibodies may in such an approach complement strain-crossrective antibodies against the CD4-binding domain (Ho *et al.*, 1991; Posner *et al.*, 1991; Thali *et al.*, 1991) in neutralization of the virus.

The B cell repertoire which is available for developing an initial immune response against HIV-1 can be evaluated by studying the ability of lymphocytes from normal, HIV-uninfected individuals to establish HIV-specific, antibody-producing cell lines *in vitro*. Accordingly, the presence of gp41/120-specific, antibody-producing cells among B lymphocytes obtained from non-infected individuals was recently confirmed and quantified using a polyclonal activation system (Zubler *et al.*, 1992). Specific B cells could be identified in 4/9 of such individuals at a frequency of 1/16000-1/49000. Since the activation system which was used in this study promoted isotype switching, the isotype of the specific B cells originally stimulated, could not be identified. It is thus not known whether these antibodies were obtained from a previously unprimed or an *in vivo* induced repertoire. The possibility cannot be excluded that these antibody specificities were raised originally in response to previous infection with other viruses known to express sequences similar to those found in HIV-1 related glycoproteins (Davis *et al.*, 1990a). Using lymphocytes from normal, uninfected individuals, gp120-specific monoclonal antibodies were also raised (paper V) against the *E. coli*-derived recombinant fragment pB1, encoding the C-terminal part of gp120, including the V3-region (Putney *et al.*, 1986). A number of LCL which specifically recognize the recombinant antigen were obtained following EBV-transformation although at a low frequency (0.06-0.3 antigen-specific LCL/10^6 lymphocytes). All antigen-specific LCL which were found in the present study produced antibodies of the IgM isotype, suggesting that they were derived from the naive/primary repertoire, and not from cells previously induced during a hyperimmune response *in vivo*. Eventually, five heterohybridomas with stable production of human antibodies recognizing the recombinant pB1 protein were established. Of these, four recognized sequences within the V3 loop of gp120. Two of them seemed to recognize non-sequential conformational epitopes, based on their recognition of solid-phase immobilized peptides. These assignments were confirmed in several cases on the basis of inhibition studies using soluble peptides. Thus, there seemed to be a immunodominant detection of the V3-loop also in the naive/primary B cell repertoire. However, the fine specificity of these antibodies did not match the fine specificity of many of the *in vivo*-induced murine monoclonal and human polyclonal antibodies, which frequently show a narrow specificity for the gly-pro-gly-arg sequence at the tip of the V3 loop (Broliden *et al.*, 1991a).

The V3-specific antibodies described above did not neutralize the virus, as results of preincubating the virus with antibody prior to their addition to the target cells indicate. Two of the antibodies, however, were able to block the spread of infection

between infected and uninfected cells in culture, implicating a potentially protective role of this type of antibody in HIV infection (paper V). The reasons why these V3-specific antibodies do not neutralize the virus is currently not understood. The affinity of the antibody may be insufficient to mediate neutralizing activity. The exact epitope specificities of the antibodies may also be improper for mediating neutralization, or the epitopes may not be sufficiently well exposed in the native antigen, as evidenced by the absence or poor recognition of such preparations. It was shown recently that other, low-affinity, V3-specific antibodies, which recognize the native antigen poorly and lack neutralizing activity tend to tolerate non-β turn conformations at the tip of the loop (Langedijk *et al.*, 1991). Apart from being devoid of functional activity, this type of antibody may represent a problem *in vivo* since antibodies which recognize the V3-loop poorly may, after antigen-binding, mediate complement-dependent enhancement of the infection rather than the neutralizing activity which is desired (Jiang *et al.*, 1991). It was recently shown, however, that virus-neutralizing antibodies can actually be established from the naive/primary repertoire, since cell lines producing human IgM antibodies which specifically recognize the tip of the V3-loop of gp120 could be developed following site-directed *in vitro* immunization (Chin *et al.*, manuscript in preparation). Furthermore, such antibodies crossreacted to a substantial degree with the principal neutralizing determinant of the divergent MN-strain, implying the possibility of establishing not only neutralizing but also crossreactive antibodies with this approach. All of these antibodies have been raised and screened against non-glycosylated recombinant or synthetic antigens. As already pointed out, conformational differences as well as differences in level of glycosylation between the recombinant antigen used in screening and the native antigen (Davis *et al.*, 1990b) probably reduce the ability of these antibodies to recognize the virus. In fact, it seems that removal of carbohydrates from the eucaryotically expressed antigen increases the binding of some of these latter V3-tip-specific human antibodies (Olofsson *et al.*, manuscript in preparation). Consequently, these monoclonal antibodies are not highly potent in neutralization assays (Hinkula *et al.*, manuscript in preparation). It has been suggested that use of native glycosylated antigens is essential for the efficient induction and identification of antibodies which recognize certain biologically relevant, crossreacting epitopes, such as the CD4-binding domain (Haigwood *et al.*, 1992; Ho *et al.*, 1991). In the future, the immunization and early screening of primary, antigen-specific clones by fully glycosylated antigen may be used in this approach for minimizing recognition of clones which show poor recognition of the native gp120 structure. This can enhance possibilities for detecting useful antibodies early in the procedure of establishing human monoclonal antibody-producing cell lines.

6.2. Antibodies against HIV-1 gp41.

Human monoclonal antibodies which recognize the procaryotically derived, recombinant protein penv9 (Putney *et al.*, 1986), expressing mainly the amino-terminal part of HIV-1 gp41 (in addition to a short sequence of the C-terminus of

gp120), were also established (paper IV) in the same way as the pB1-specific antibodies described above. A major immunogenic epitope of HIV-1 resides within this sequence of gp41. This epitope has been shown to induce antibodies in a great majority of infected individuals (Broliden *et al.*, 1991b; Gnann *et al.*, 1987). Functional activities, including virus neutralization (Broliden *et al.*, 1992), ADCC (Tyler *et al.*, 1990), the killing of infected cells by toxin-conjugated antibodies (Till *et al.*, 1989) and the complement-dependent enhancement of virus infection (Robinson *et al.*, 1990a, 1990b, 1991) have also been shown to be mediated by antibodies which recognize sequences within penv9. In addition, the presence of antibodies against a putative immunosuppressive part of gp41 has been shown to correlate with the health status of HIV-infected individuals (Klasse *et al.*, 1988). Such findings implicate the importance of an understanding of these epitopes for proper development of diagnostic reagents as well as vaccines and antibody-based therapeutics. As implied by the highly immunogenic nature of gp41, possibly induced by crossreating epitopes expressed by other viruses (Davis *et al.*, 1990a), a number of human monoclonal antibodies which recognize the conserved, immunodominant epitope expressed by synthetic peptides (expressing e.g. aa residues 579-604) have been established from lymphocytes derived from HIV-1 infected individuals (Banapour *et al.*, 1987; Bugge *et al.*, 1990; Gorny *et al.*, 1989; Tyler *et al.*, 1990).

The monoclonal antibodies which recognize the recombinant protein penv9 and are produced by hybridomas obtained following immortalization of lymphocytes from non-infected donors (paper IV) were all of the IgM isotype, suggesting that they originated from the naive/primary immune repertoire. Three other IgM antibodies which recognize gp41, have been established under similar conditions in an independent study (Pollock *et al.*, 1989). One of the antibodies described in paper IV, MO30 (also designated H2), has been investigated in some detail. The amino acid sequence of the heavy and light chain variable domains have been deduced following PCR amplification of cDNA (Borrebaeck *et al.*, 1990; Larrick *et al.*, 1989a). These sequences seem to be of a non-germline type, although the Vκ-domain of MO30 was highly homologous with the sequence occurring in the digoxin-specific antibody MO6 (paper III). Following the immunization of mice, polyclonal and monoclonal anti-idiotypic antibodies against MO30 have been obtained. These anti-idiotypic reagents detect idiotopes present in the sera of HIV-infected but not in sera obtained from uninfected individuals. This idiotope is not restricted to gp41-specific antibodies, but can be found on anti-gp120 antibodies in the polyclonal sera of infected individuals (Müller *et al.*, 1991). It seems that the normal human antibody repertoire thus contains clones expressing idiotopes which are not extensively utilized in the non-HIV-infected individual, but which after HIV-infection are specifically utilized in the antibody response against HIV-related proteins. However, in contrast to the antibodies descibed above, which were obtained from infected donors (Banapour *et al.*, 1987; Bugge *et al.*, 1990; Gorny *et al.*, 1989; Tyler *et al.*, 1990), these antibodies from the naive/primary immune repertoire seem to recognize a different conformational epitope (paper IV). A similar difference between *in vivo*- and *in vitro*-induced antibodies was apparent for the V3-specific antibodies, as described in

section 6.1. These differences, observed at the level of the epitopes which are preferentially detected by the naive and induced immune repertoire, accord with previously reported observations concerning murine anti-tubulin antibodies (Matthes *et al.*, 1988), as mentioned above. However, since the *in vitro*-induced (paper IV, V) and *in vivo*-induced gp41/120-specific antibodies have been developed using dissimilar immunogen/antigen preparations and assay systems, the possibility cannot not be completely excluded that the observed differences depend to some extent on the preferential exposure of different specificities in the various sets of antigens.

7. The immune response against cytomegalovirus-derived proteins.

Human cytomegalovirus (CMV) is a member of the betaherpesvirus subfamily of the herpesviruses. It is thus distantly related to a number of other pathogenic viruses such as herpes simplex virus, EBV and Varicella-Zoster virus. This large DNA-virus is characterized among other things by its narrow host range and its relative restriction to fibroblasts for growth *in vitro*, although some other target cells, such as primary brain cells and cell lines of nervous tissue origin may also promote viral replication *in vitro* (Wroblewska *et al.*, 1981). *In vivo* infection results in a life-long carrier state in infected individuals, although infection is generally without any long-term clinical manifestations to an immunocompetent individual. Congenital CMV-infection, however, is a major cause of a variety of neurological abnormalities (Ahlfors, 1982; Pass *et al.*, 1991), indicating the importance of tools for minimizing the hazardous outcome of such a viral infection. Furthermore, although representing no problem to a normal adult, CMV-infection or reactivation of latent CMV often leads to serious clinical conditions in immunocompromised individuals (Pass *et al.*, 1991), implying that immunological control is an important factor in suppressing the progression of the virus *in vivo*. The development of vaccines based on whole live virus prparations and on isolated viral components has been undertaken (Gönczöl & Plotkin, 1990), and therapy with immunoglobulin preprations which contain cytomegalovirus-specific antibodies has been suggested to improve the clinical condition of immunocompromised patients (Meyers *et al.*, 1983). Although antibody therapy is frequently unable to completely block viral activity, it seems able to reduce the severity of the disease (Snydman, 1991; Winston *et al.*, 1987), implying that virus-specific antibodies do play a role in the control of the infection *in vivo*. Very little is known, however, regarding the mechanisms behind these effects. The immunological response against CMV has been extensively assessed in humans both with respect to the polyclonal humoral response and to the cellular response (Rasmussen, 1990). It has been shown *in vitro* that antibodies may neutralize the infectivity of cell-free virus (Britt, 1984; Kari *et al.*, 1986; Rasmussen *et al.*, 1984; Utz *et al.*, 1989)) and reduce the spread of the virus between infected cells (Tomiyama & Masuho, 1990). Complement-dependent cytotoxicity (Betts & Schmidt, 1981; Middeldorp *et al.*, 1986) towards infected cells has also been suggested to be a mechanism which protects the infected but healthy individual. In addition to antibody-mediated protective effects, NK-cell mediated cytotoxicity (Borysiewicz *et al.*, 1985) and antigen-specific MHC-restricted T-cell mediated

cytotoxicity (Borysiewicz *et al.*, 1983, 1988; Lindsley *et al.*, 1986) have been shown to aid in the killing of CMV-infected target cells.

The large genome of CMV (approximately 230 kb) has the potential of expressing a large number (>200) of proteins of which at least 35 occur in the intact virus paticle (Chee *et al.*, 1990; Landini & Michelson, 1988). Of these, the major glycoproteins occurring in the envelope of the virus, are of major interest as targets for antibody therapy *in vivo*, while the most immunogenic and commonly occurring proteins are important targets to investigate for diagnostic purposes.

7.1. Antibodies against the pp65 lower matrix protein of human CMV.

CMV strains passed several times *in vitro* will strongly overproduce a viral antigen having a molecular weight of approximately 65 kDa. This protein (pp65), which may constitute >90 % of the CMV-encoded proteins found in dense body particles (Irmiere & Gibson, 1983; Roby & Gibson, 1986) and approximately 15% of the total viral protein synthesized (Rasmussen, 1990) following infection with laboratory strains of CMV, comprises only a minor part of the protein present in clinical isolates of CMV. It is present in the matrix of the virus (the volume between the viral capsid and the outer membrane) (Gibson, 1991) and not being exposed on the surface of the intact virus or of the infected cells, it is not a target for neutralizing antibodies (Britt and Vugler, 1987). However, it is a major target for CMV-specific T-cells, suggesting its importance in the development in the immune response against CMV (Forman *et al.*, 1985). Due to its extensive presence in *in vitro* produced CMV-antigen preparations, it is also an important part of many assay systems designed to evaluate serological levels of CMV-specific antibodies. In addition, this antigen has recently been shown to be a very useful marker in the CMV antigenimia assay used to make the diagnosis of active CMV infection (Grefte *et al.*, 1991; Revello *et al.*, 1992; The *et al.*, 1991).

The gene encoding the major matrix protein of pp65 has been cloned and sequenced (Nowak *et al.*, 1984a; Pande *et al.*, 1984, 1991; Rüger *et al.*, 1987). Its sequence coincides with a purified protein described by Clark *et al.* (1984), and it differs from that of protein(s) of similar molecular weight suggested to bind neutralizing antibodies (Britt & Auger, 1985; Davis & Huang, 1985; Davis *et al.*, 1984). The gene product is posttranscriptionally modified by phosphorylation (Gibson, 1983; Britt & Vugler, 1987), but the presence or absence of carbohydrates on this antigen is a matter of controversy (Clark *et al.*, 1984; Britt & Vugler, 1987).

In initial experiments, CMV-specific LCL and hybridoma cell lines were screened for antibodies which recognize a membrane extract derived from CMV-infected cells (paper VI). Of the IgG-producing clones which were established, 4/5 (MO53, MO58, MO61 and MO79) produced antibodies which recognized the pp65 antigen. All of these antibodies were of the IgG1 isotype, in agreement with the dominant subclass response against CMV *in vivo* (Gilljam & Wahren, 1989). Whether pp65-related

specificities are truly dominant among the CMV-specific peripheral B cells, which can be immortalized by EBV, or the outcome of the immortalization and screening process is a consequence of the assay system which may favor detection of antibodies against this abundant antigen, is not known. Similar results, with a majority of the clones recognizing the pp65 antigen, have been obtained by others, using a similar screening system based on unpurifed viral antigen (Tomiyama *et al.*, 1990). In fact, a very large number of the CMV-specific human monoclonal antibodies which have been reported recognize this antigen (paper VI; Alexander *et al.*, 1988; Bron *et al.*, 1990; Foung *et al.*, 1989; Gustafsson *et al.*, 1991; Larose *et al.*, 1991; Redmond *et al.*, 1986; Tomiyama *et al.*, 1990), suggesting that infected individuals mount a strong immune reaction against this antigen *in vivo*.

The repertoire of human antibodies recognizing pp65 has been shown to detect at least four different epitopes, of which three are overlapping (paper VI). One of the human antibodies (MO58) recognizes an apparently linear epitope between aa 283 and 288 as determined by peptide mapping (paper VI; Geysen *et al.*, 1984). The epitope assignment is in agreement with the observed specific binding of MO58 to the recombinant protein pHE68-1 (covering residues 184-379 of pp65) (Mach & Plachter, personal communication; Plachter *et al.*, 1990). It appears, however, that this particular epitope is not a major determinant of pp65 since only a few CMV-seropositive human sera are able to detect this or closely related epitopes, and since the intensity of any detectable reaction (as determined by the extent of color development in immunoblotting) is low (Plachter *et al.*, 1990). In addition, this epitope is not detected frequently by murine monoclonal antibodies (Plachter *et al.*, 1990), although at least one mouse antibody (C5FII; kindly provided by Dr. V.-A. Sundqvist) has been shown independently to recognize an epitope spatially related to the epitope recognized by MO58 (data not shown). This demonstrates the fact that this or a closely related epitope can induce a humoral immune response not in only the human but also the mouse immune system.

Although mouse antibodies recognize at least three distinct epitopes within pp65 (Revello *et al.*, 1992), it seems that sequences between aa 401 and 470 (in particular between residues 401 and 426) of pp65 are immunodominant in mice. In fact, these sequences were recognized by 14/14 previously studied mouse monoclonal antibodies (Plachter *et al.*, 1990). In addition, some CMV-seropositive human sera show strong binding to this recombinant protein in Western blots (Plachter *et al.*, 1990). None of the human monoclonal antibodies described here, however, detect these recombinant proteins following SDS-PAGE (Mach & Plachter, personal communication). Another study of the binding of polyclonal IgG-antibody to recombinant proteins of pp65 showed a higher frequency of recognition of the sequence 297-458 by CMV-seropositive sera, than of the 401-470 sequence described above, at least among individuals with high IgG-titers against CMV (Landini *et al.*, 1990). This indicates the presence of additional epitopes requiring residues between aa 297 and 400. Although it has been reported that all recombinant fragments of pp65 recognized by the human antibody repertoire were located in the C-terminal part of the protein (Lindenmaier *et al.*, 1990), an N-

terminal epitope defined by the human antibody Z01 was found recently to be located between aa residues 108 and 253 of pp65 (Bradshaw *et al.*, 1991). The linear or conformational nature of this epitope and its relation to the epitope recognized by MO53, MO61 and MO79 has not been adequately investigated, however.

It has been suggested that the immune response to pp65, as detected by Western blot, is relatively weak or undetectable in many CMV-seropositive individuals (Gilljam and Wahren, 1989; Jahn *et al.*, 1987; Zaia *et al.*, 1986). Also, the frequency with which sera recognize recombinant fragments of the CMV-related protein pp150 is higher than that for recognition of recombinant pp65 (Landini *et al.*, 1990; Plachter *et al.*, 1990), suggesting that the antibody response to the latter antigen is poorly developed. This could be partly explained by the low level of expression of pp65 in clinical isolates of CMV, which may be unable to effectively induce an immune response in the infected individual (Jahn *et al.*, 1987). In rabbits it has been shown that immunization with purified pp65 induces a strong immune response as determined by western blot, indicating that the protein, if presented properly to the immune system, is immunogenic (Jahn *et al.*, 1987). In view of the high frequency of human monoclonal antibodies recognizing pp65 (paper VI; Tomiyama *et al.*, 1990) and the ability of several human antibodies to recognize eucaryotically-derived recombinant pp65, but not fragments expressed in *E. coli* (Mach & Plachter, personal communication), it can be speculated that the human humoral immune response to pp65 detects mainly epitopes which are highly dependent on the native conformation. It has been suggested independently that denaturation of the antigen severely impairs the binding of human polyclonal pp65-specific antibodies (Zaia *et al.*, 1986). The use of techniques dependent on non-native antigens or denatured pp65 (such as SDS-PAGE) may thus underestimate the level of pp65-specific antibodies present. The recognition of conformational epitopes in pp65 is supported by the fact that 3/4 of the antibodies could not be properly mapped to a single linear sequence by peptide mapping. Furthermore, the human monoclonal antibodies (paper VI) all required very sensitive detection systems to show binding to the denatured native antigen following SDS-PAGE (data not shown) while efficiently precipitating the native antigen. One of these epitopes, recognized by MO53, could be at least partly identified as being comprised of two peptide sequences, as determined by peptide binding assays. One of these peptides is a part of the recombinant fragment recognized by the human antibody Z01 described above (Bradshaw *et al.*, 1991), but it is not known whether these antibodies actually recognize overlapping epitopes in the native antigen. The epitopes recognized by two antibodies (MO61 and MO79) could not be mapped further by the peptide mapping technique (paper VI). In addition, the partly overlapping epitopes recognized by these antibodies were particularly sensitive to freeze-thaw cycles, indicating that they were highly conformation-dependent. On the basis of these data it can be suggested that a substantial part of the humoral immune response to CMV pp65 recognizes conformational epitopes. Future studies of the relative importance of the various epitopes in the total immune response to pp65, and identifying the conformational epitopes of pp65 will provide important information useful in the

selection of recombinant proteins from pp65 which can be utilized as tool in the serodiagnosis of CMV infection (Landini *et al.*, 1990; Lindenmaier *et al.*, 1990; Plachter, *et al.*, 1990).

7.2. Antibodies against cytomegalovirus glycoproteins.

Human CMV expresses several glycoprotein complexes in its envelope. These proteins have been biochemically described in some detail (Gretch *et al.*, 1988b). Antibodies to these structures have been detected in the serum of infected donors and immunized animals (Liu *et al.*, 1988; Nowak *et al.*, 1984b; Pereira *et al.*, 1982; van der Voort *et al.*, 1989) and polyclonal and monoclonal antibodies with such specificities have been shown to neutralize the infectivity of the virus. Briefly, glycoprotein complex I (gC-I), which is comprised of two disulphide-linked proteins (Gibson, 1991), is the major target for neutralizing antibodies in human seropositive sera (Britt *et al*. 1990). Neutralizing activities have also been identified among antibodies which recognize gC-II, a complex comprised of several different proteins, of which at least some are encoded by the HXLF multigene family (Gretch *et al.*, 1988a; Kari & Gehrz, 1988; Kari *et al.*, 1990a; Weston & Barrell, 1986). Certain epitopes recognized by anti-gC-II specific antibodies seem to be conserved among herpesviruses, including herpes simplex virus (Kari *et al.*, 1986), suggesting these proteins to have an important function for the viruses. An inverse correlation of antibodies to this glycoprotein complex and congenital infection in infants appears to exist, suggesting a role of gC-II specific antibodies in the defense against CMV (Kari & Gehrz, 1990). Furthermore, murine antibodies specific for gC-III (containing the 86 kDa gH protein) have been shown to mediate virus neutralizing activities either in a strain-crossreactive (Cranage *et al.*, 1988; Gretch *et al.*, 1988b; Rasmussen *et al.*, 1984) or in a strain-specific (Urban *et al.*, 1992) manner. In addition, the establishment of a human, gC-III-specific neutralizing antibody (Ehrlich & Östberg, 1990), and the detection of neutralizing antibodies recognizing this antigen in human sera (Urban *et al.*, 1992), implicate a protective role of this specificity in the humoral immune surveillance of human CMV *in vivo* as well.

CMV glycoproteins belonging to the gC-I complex (gp58/116; gB) are synthezised as a high molecular weight precursor. After substantial posttranslational modifications involving O- and N-linked glycosylation (Benko & Gibson, 1986; Britt & Vugler, 1989), this molecule is cleaved into a high molecular weight N-terminal fragment (gp116) and a low molecular weight, transmembrane, C-terminal fragment (gp58) (Britt & Auger, 1986; Kari *et al.*, 1990b; Meyer *et al.*, 1990; Gibson, 1991). This process has been verified in CMV strain Towne and been implicated in the Ad-169 strain, which has the same type of cleavage site (Spaete *et al.*, 1988). The N-terminal signal peptide of the precursor is also removed by proteolytic cleavage. The two proteins formed by these processes are linked together by disulphide bonds in the mature complex, which is present in the virus envelope. Being a major component of the envelope proteins, it is a potential target for neutralizing antibodies. The importance of this antigen for viral function is implicated by the

observation that certain features of gB, such as certain amino acid sequences, certain sites for N-linked glycosylation and a number of proline and cystein residues, are highly conserved among different herpesviruses (Borchers *et al.*, 1991; Cranage *et al.*, 1986). In addition, the gB protein, or its equivalent, has been shown to have important functions in the process of virus- and cell-membrane fusion (Fitzpatrick *et al.*, 1990; Navarro *et al.*, 1992; Peeters *et al.*, 1992).

A number of reagents have been developed for investigating the immune response to gp58/116. Purified protein complexes have been shown in humans to induce polyclonal antibodies which neutralize the virus (Gönczöl *et al.*, 1986; Gönczöl & Plotkin, 1990). Recombinant gp58/116 expressed in a vaccinia virus vector has also been shown to induce neutralizing antibodies *in vivo* (Britt *et al.*, 1988). Furthermore, mouse and human monolonal antibodies to gp58 (paper VII; Banks *et al.*, 1989; Britt, 1984; Ehrlich and Östberg, 1990; Emanuel *et al.*, 1988; Foung *et al.*, 1990a; Lussenhop *et al.*, 1988; Masuho *et al.*, 1987; Pereira & Hoffman, 1986; Rasmussen *et al.*, 1985; Tomiyama *et al.*, 1990; Utz *et al.*, 1989) and gp116 (paper VII; Kari *et al.*, 1990b; Masuho *et al.*, 1987; Meyer *et al.*, 1990, 1992) have been shown to mediate neutralizing activities against CMV *in vitro*. A complete outline of the known B cell epitopes in gp58/116, including epitopes which do not bind neutralizing antibodies, has been presented recently to account for the reactivities obtained for such antibodies (Mach & Britt, 1991)

Many mouse monoclonal antibodies against gp58 seem to recognize epitopes involving sequences between aa residues 608 and 625 (Utz *et al.*, 1989). Further studies have implied sequences between residues 589 and 645 (Kniess *et al.*, 1991), between residues 461 and 680 (Banks *et al.*, 1989), between residues 557 and 635 (Wagner *et al.*, 1992) and within 34 and 43 kDa proteolytic fragments of gp58, or more specifically between residues 514 and 635 (Kari & Gehrz, 1991), to be important for antibody binding. The binding of human monoclonal antibodies to overlapping epitopes within this region required the presence of residues 549 to 635, 552 to 630 or 552 to 635 depending on the antibody (table 4; paper VII; Wagner *et al.*, 1992) This agrees closely with results obtained for polyclonal human antibodies (Wagner *et al.*, 1992). It is also in close agreement with CNBr-cleavage studies, which have suggested that sequences close to or between residues met^{541}, met^{564} and/or met^{635} (Ad-169 sequence) are important for antibody binding to the antigen (Kari & Gehrz, 1991). The fine specificities of the antibodies imply that several closely related, partially overlapping epitopes are recognized by the mouse and human immune systems. Based on mapping using solid phase immobilized peptides, each of several of the human monoclonal antibodies seemed to recognize similar sequences in a discontinous epitope, including amino acids between residues 570-579 and 606-619 (paper VII). The discontinous nature of the epitope does not preclude the recognition of denatured antigen following SDS-PAGE (paper VII), however. Furthermore, there was no absolute correlation between the ability to recognize individual synthetic peptides and the potency of these human antibodies to recognize denatured antigen following SDS-PAGE and western blot. These results indicate that some of the mouse antibodies previously thought to recognize continous epitopes

based on recognition of denatured antigen (Kari & Gehrz, 1991; Lussenhop *et al.*, 1988) may in fact recognize discontinous epitopes just as the human antibodies described above do.

Neutralizing antibodies specific for gp58/116 may or may not require complement in order to prevent infection of the target cell. It has been suggested that procaryotically derived recombinant gp58/116 will mainly induce antibodies mediating complement-independent neutralization in mice. In contrast, the eucaryotically derived counterpart mainly induces complement-dependent neutralizing antibodies (Britt *et al.*, 1988). It is not clear, however, whether the complement-dependent and independent antibodies raised in those experiments recognize epitopes which are overlapping (such as the epitopes in gp58) or entirely unrelated, but it is clear that any virus-neutralizing activity of human antibodies which recognize the gp58-related *E. coli*-derived recombinant fragment, is independent of complement (paper VII).

At least two continous epitopes have been identified within the N-terminal part of gp116, one between residues 67 and 86 which is isolate crossreactive (paper VII; Meyer *et al.*, 1990, 1992; Tomiyama & Masuho, 1990) and one between residues 50 and 54 which is isolate specific (Meyer *et al.*, 1992). These differences in crossreactivity are in agreement with the known sequence variability in the most N-terminal part of gp116 and the more conserved nature of the residues following aa 68 (Cranage *et al.*, 1986; Spaete *et al.*, 1988). Both human and mouse antibodies which recognize the strain-crossreactive epitope of gp116 seem to be independent of the presence of complement in mediating neutralization (paper VII; Kari *et al.*, 1990b; Meyer *et al.*, 1990, 1992). Antibodies recognizing this epitope of gp116 may be particularly useful for therapeutic applications due to their crossreactive nature, their complete independence of complement activation for neutralization, and their high potency. Clinial trials have been initiated recently using such a gp116-specific human monoclonal antibody. Initial studies show this human antibody to have a substantially longer half-life *in vivo* than murine antibodies do. Furthermore, the very low toxicity of this antibody (Masuho *et al.*, 1990; Azuma *et al.*, 1991) and another human CMV-specific antibody (Drobyski *et al.*, 1991), suggests that therapeutics based on human monoclonal antibodies are an attractive alternative to murine monoclonal antibodies as well as to the polyclonal immunoglobulin preparations currently available.

8. Discussion, general conclusions and future prospects.

* The outgrowth of EBV-immortalized human B cells is facilitated by pretreatment of the mononuclear cell preparation with L-leucyl-L-leucine methyl ester. This treatment procedure complements other previously established techniques used to allow establishment of LCL from lymphocytes obtained from EBV-infected donors. The effect of this dipeptide ester on the reduction of LCL outgrowth inhibition seems to involve depletion or functional impaiment of the effector

function normally present within both CD4+ and CD8+ T cell populations. Accordingly, the ability of CD4+ T cells to respond to autologous EBV-infected B cells by the production of γ-IFN-specific mRNA is substantially reduced.

* A variety of techniques to develop antibodies from the human immunoglobulin repertoire are available today. By combining such approaches (e.g., as in figure 2) the procedure can be optimized to deal with the different experimental problems that may occur. In particular the use of combinatorial libraries in association with affinity maturation technology may provide vital approaches to overcome the problems which are still associated with the LCL/hybridoma technology currently employed.

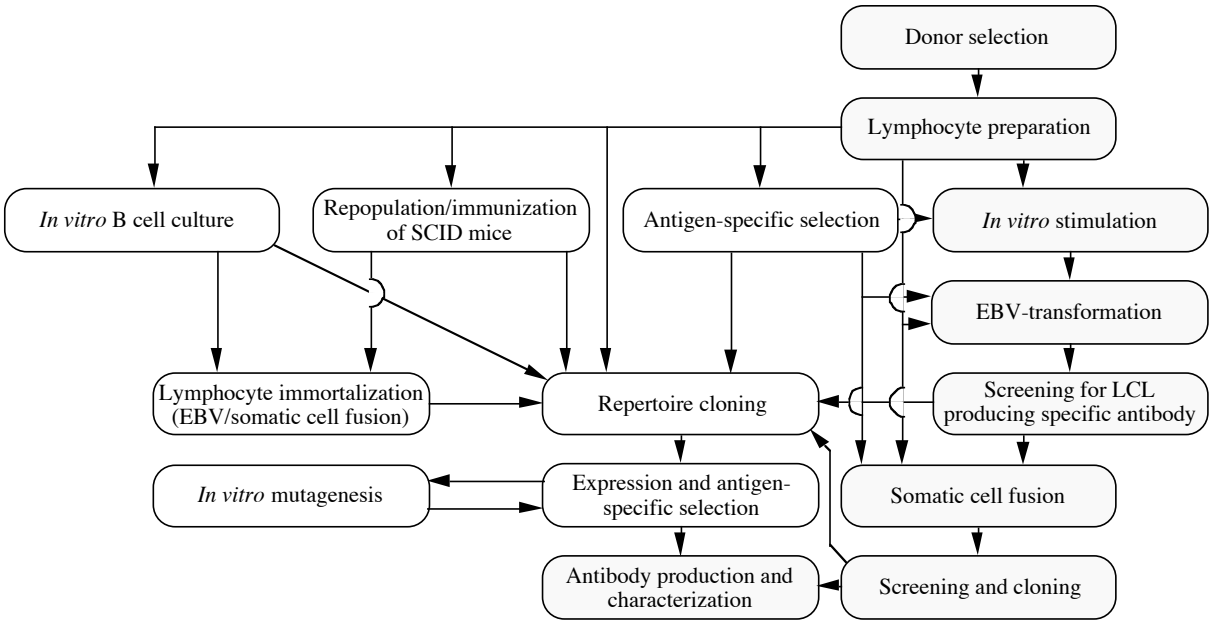

Figure 2. Flow chart describing procedures useful in the establishment of e.g. cell lines producing human monoclonal antibodies, using specificities from the immunoglobulin repertoire established in human lymphocyte donors. The grey boxes describe procedures that have been used in the work presented in this thesis (paper I-VII).

* Cell lines producing human monoclonal antibodies can be established from lymphocytes obtained from donors which have not been exposed to the antigen studied. The generation of such cell lines is in agreement with recent observations indicating the presence of such specific B cells in seronegative donors (Zubler *et al.*, 1992). Similarly, combinatorial libraries established from non-immunized individuals are able to provide genetic information encoding the antibody specificities which recognize the antigen (Marks *et al.*, 1991). However, whether the specificities obtained from these combinatorial libraries are actually present in the *in vivo* repertoire or represent novel heavy and light chain combinations occurring as a consequence of the scrambling of the genes, is not known. The antibodies obtained by these procedures are generally of the IgM isotype (or can be developed primarily from IgM-derived combinatorial

libraries) and frequently have a low affinity for the antigen, suggesting that they originate from the naive repertoire. Despite these characteristics, functional effects, including virus neutralization, have been shown to be mediated by some of the antibodies which specifically recognize the principal neutralizing loop of HIV-1. Whether such antibodies play may a role in the primary infection process *in vivo* remains to be evaluated.

* The future development of techniques which may possibly allow the generation of hyperimmune human antibody repertoires to occur either *in vivo* (e.g., in SCID-hu mice or in mice transgenic for the human heavy and light chain locuses) or *in vitro* (in culture systems allowing not only expansion of, but also affinity maturation and selection of preexisting repertoires) will be invaluable. Such methods will permit the efficient establishment of cell lines producing useful human antibodies. In addition, genetic engineering and combinatorial display libraries may be able to improve antigen recognition and functional activities of the low-affinity antibodies which will continue to be a substantial portion of the repertoires which are available from non-immunized donors.

* Human monoclonal antibodies developed from the naive/primary immunoglobulin repertoire recognize sets of epitopes which are not always identical with the major reactivities observed to arise as a consequence of *in vivo* immunization. This phenomena may be used to develop antibodies with otherwise inaccessible (not immunodominant) specificities, much in the same way as the human antibody repertoire may recognize epitopes (such as blood group D) towards which a murine immunoglobulin response cannot be developed.

* A number of human monoclonal antibodies which seemingly recognize conformational peptide epitopes have been developed. The conformational nature of the epitope has in some cases been identified by the inability of the antibody to bind synthetic overlapping short (nine to twenty residues) peptides, by the denaturation-sensitive nature of the epitope and/or by antibody recognition of sequences being located far apart in the primary sequence of the antigen. Similar observations of antibody binding to individual peptides which are separated in the amino acid sequence but which together make up the intact epitope have been reported earlier (McGuinness *et al.*, 1990; Meloen *et al.*, 1991 Parry *et al.*, 1989). Some peptide epitopes (e.g., as recognized by antibodies MO58 and ITC88) have been described as linear, as based on data showing specific interaction between these antibodies and one single short peptide structure. The existence of truly linear epitopes, however, may be questioned due to the absence of sufficiently large (approximately 700 Å2 (Janin & Chothia, 1990)) "continous" protein surfaces, i.e. surfaces only containing atoms from adjacent amino acid residues, in native proteins. The linear epitopes described may, thus, just represent the only crossreacting sequence that is able to mimic the epitope sufficiently well (Barlow *et al.*, 1986; van Regelmortel, 1990). Further refinement using synthetic, amino acid substituted peptides or mutated

peptides expressed in phage display libraries (Meloen *et al.*, 1991) may serve to at least partly identify the most important residues involved in the antigen-antibody interaction.

* The use of entirely solid-phase peptide-based epitope assignment (for example as described by Geysen *et al.*, 1984) may be associated with problems related to non-specific interactions between peptides and antibodies (Horsfall *et al.*, 1991). As reported recently, peptide based epitope mapping may, furthermore, suffer from peptide batch variability (Halsey *et al.*, 1992), indicating the need for caution in interpreting results obtained by such procedures. For these reasons epitope assignments in the present study have insofar as possible been tested and confirmed by alternative methods (liquid phase blocking using synthetic peptides or binding to recombinant protein fragments) to ensure as correctly assigned epitopes as possible.

* Human monoclonal antibodies recognizing the major matrix protein (pp65) of CMV frequently recognize conformational epitopes. Since a similar antibody repertoire may occur in polyclonal human sera, this has implications for the selection of recombinant pp65-related antigens for use in the sero-diagnosis of CMV infection.

* A variety of fine specificities among human antibodies which recognize the previously defined major neutralizing epitope of CMV gp58 have been detected. Of particular importance is the fact that the different fine specificities of the different clonotypes of the human immunoglobulin repertoire could not easily have been detected by other techniques. At the level of refinement, there is no absolute correlation in this system between epitope designation and the ability of the antibody to neutralize the virus, although the recognition of a minimal epitope covered by residues 552-630 seems to be related to neutralizing activity. The lack of correlation between neutralizing activity and the binding to an epitope located between residues 552 and 635 (human antibodies) and between residues 557 and 635 (mouse antibodies) (table 4) is not well understood. It cannot be excluded, however, that the affinity constant of the antibodies for the native antigen and the kinetics of antigen-antibody association/dissociation may play a role in determining the ability of each of the antibodies to neutralize the virus. The usefulness of the neutralizing gB-specific antibodies described in paper VII for passive immunotherapy remains to be fully evaluated. Their ability to recognize and neutralize diverse clinical isolates of CMV is currently under investigation. Combinations of isolate-crossreactive antibodies specific for gB with antibodies recognizing other CMV-related antigens and possibly with antiviral drugs such as ganciclovir, may eventually enable development of potently neutralizing immunoglobulin preparations which can be used for safe and efficient treatment of CMV-related diseases.

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Table 1. Problems initially encountered in human monoclonal antibody technology.

1. Difficulties in obtaining suitable immune lymphocytes.
2. Instability of EBV-transformed lymphoblastoid cell lines.
3. Few available cell lines suitable as fusion partners.
4. Low immortalization frequencies in somatic cell fusion.
5. Instability of human chromosomes in mouse cell lines.
6. Low antibody production of many LCL and hybridomas producing human monoclonal antibody.

Table 2. List of techniques used to increase the frequency of antigen-specific human immune B cells or to by other means create a desired human immunoglobulin repertoire or human-like antibodies.

1. *In vivo* immunization.
2. Antigen-specific selection of B-cells carrying surface immunoglobulins of the desired specificity.
3. Removal of B lymphocytes carrying surface immunoglobulin following internalization of surface immunoglobulin on antigen-specific B cells.
4. Immunization of SCID mice repopulated with human lymphocytes.
5. Culture of human B-cells *in vitro*.
6. *In vitro* differentiation of human B cell precursors.
7. *In vitro* immunization/stimulation.
8. Creation of transgenic mice carrying unrearranged human immunoglobulin loci.
9. Creation of recombinant, combinatorial libraries, mainly in phage.
10. Hypermutation of immunoglobulin genes in *in vitro* or *in vivo*.
11. Humanizing mouse antibodies with human-constant regions or transplanting the CDRs into an entire human immunoglobulin framework.

Table 3. List of human monoclonal antibodies described in this investigation.

Clone number	Heavy chain	Light chain	Specificity	Reference
MO6	μ	κ	digoxin, digitoxin	III
LH92	μ	λ	digoxin	III
LH114	μ	κ	digoxin, digitoxin, ouabain	III
MO28	μ	κ	penv9	IV
MO30	μ	κ	penv9	IV
MO43	μ	κ	penv9	IV
MO86	μ	κ	pB1	V
MO96	μ	κ	pB1	V
MO97	μ	κ	pB1	V
MO99	μ	κ	pB1	V
MO101	μ	κ	pB1	V
MO53	γ1	κ	CMV pp65	VI
MO58	γ1	κ	CMV pp65	VI
MO61	γ1	λ	CMV pp65	VI
MO69	μ	λ	CMV	VI
MO79	γ1	κ	CMV pp65	VI
MO80	γ1	κ	CMV p35	VI
ITC33	γ1	λ	pMbg58/CMV gp58/130	VII
ITC34	γ1	κ	pMbg58	VII
ITC39	γ1	λ	pMbg58/CMV gp58/130	VII
ITC48	γ1	κ	pMbg58/CMV gp58/130	VII
ITC52	γ1	κ	pMbg58/CMV gp58/130	VII
ITC63B	γ1	λ	pMbg58/CMV gp58/130	VII
ITC63C	γ1	λ	pMbg58/CMV gp58/130	VII
ITC88	γ1	κ	pHM90-5/CMV gp116/130	VII

Table 4. Sets of epitopes recognized by human and murine antibodies specific for the major neutralizing epitope in CMV gp58, and the ability of these antibodies to neutralize the virus.

Antibody	Origin	Neutralizing activity	Minimal epitope (aa residues)[a]a
ITC33 [b]	human	no	549-635
ITC39 [b]	human	no	552-635
ITC52 [b]	human	yes	552-635
ITC63B [b]	human	yes	552-635
ITC63C [b]	human	yes	552-635
ITC48 [b]	human	yes	552-630
[c]	human	yes	552-630
27-287 [c]	mouse	no	557-635
27-156 [c]	mouse	no	557-635
7-17 [c]	mouse	yes	557-635
polyclonal sera	human	yes	557-630

[a] determined by antibody reactivity to recombinant fragments of gp58.
[b] paper VII.
[c] Wagner *et al.*, 1992.

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